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Repaglinide inhibits cyclosporine A-induced renal tubular toxicity by affecting apoptosis and *Bax* and *Bcl-2* expression

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Background/aim: Repaglinide (RG) is a prandial glucose regulator used for the treatment of type 2 diabetes. Our recent study showed that RG plays an important role in cyclosporine A (CsA)-induced nephrotoxicity through its antioxidant properties. However, it is not known whether or not RG has any protective effect on CsA-induced renal tubular toxicity by affecting apoptosis and expression of apoptosis-associated protein. The purpose of this study was to investigate the effects of RG on CsA-induced renal tubule apoptosis and *Bax* and *Bcl-2* protein expression in rats.

Materials and methods: Forty male Sprague Dawley rats weighing 250–300 g were randomly divided into four groups: administrations of olive oil (control, per os (PO)), CsA (30 mg/kg in olive oil, subcutaneous (SC)), and RG (0.2 or 0.4 mg/kg, PO) plus CsA (30 mg/kg in olive oil, SC) every day for 15 days.

Results: Our results showed that SC administration of CsA (30 mg/kg) to rats produced marked injury and apoptosis, elevation of *Bax* protein expression, and inhibition of *Bcl-2* protein expression in the kidneys, which were reversed significantly by oral administration of RG (0.2 or 0.4 mg/kg).

Conclusion: The findings of our study indicate that RG may play an important role in protecting the kidneys through inhibiting apoptosis, *Bax*, and *Bcl-2* protein expression.

Key words: Repaglinide, cyclosporine A, apoptosis, *Bax* and *Bcl-2* expression, renal, rat

1. Introduction

Repaglinide (RG) is an oral antihyperglycemic agent used for the treatment of noninsulin-dependent diabetes mellitus. It belongs to the meglitinide class of short-acting insulin secretagogues, which act by binding to β cells of the pancreas to stimulate insulin release (1). It has been reported that RG treatment decreases the concentration of lipid hydroperoxide (LPO) and increases the activity of superoxide dismutase (SOD) in kidneys of diabetic nephropathy (2–6). Cyclosporine A (CsA) is a drug used to treat many autoimmune diseases and to prevent transplant rejection. Nephrotoxicity constitutes its main adverse effect and can cause acute or chronic kidney damage (7). Many studies demonstrated that CsA-induced nephrotoxicity is mediated by oxidative damage (8–10). Our recent study showed that RG plays an important role in CsA-induced nephrotoxicity through its antioxidant properties (11).

Other studies showed that CsA-induced cytotoxicity was related to apoptosis in renal tubular or endothelial cells (12,13). It is not known, however, whether RG has any effect on CsA-induced renal tubular apoptosis by affecting the expression of apoptosis-associated proteins. Therefore, the purpose of the present study was to investigate the effects of RG on CsA-induced renal tubular apoptosis, and the expression of *Bax* and *Bcl-2* proteins in rats.

2. Materials and methods

2.1. Animals and treatment

Adult male Sprague Dawley rats weighing 250–300 g were used. The animals were obtained from the Experimental Research Centre, Wuhan, China. The animals were maintained at 25 °C in a room with a 12/12 light/dark cycle and had free access to water and food.

Forty rats were randomly allocated into 4 groups (n = 10). Olive oil (1 mL) was administered by gavage (IG) to

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the first (normal control) group every day for 15 days, and in the second (CsA model control) group, the rats were given subcutaneous (SC) CsA (Novartis Pharma GmbH, Mannheim, Germany) injection, 30 mg/kg in olive oil daily for 15 days. The CsA rats of the third and the fourth groups received RG (Sigma, St. Louis, MO, USA) (IG) at doses of 0.2 mg/kg and 0.4 mg/kg daily for 15 days, respectively. To prevent a RG-induced rat hypoglycemia reaction, 5% glucose administration by IG after per os RG was given daily during the experimental period.

At the end of day 15, animals in all groups were anesthetized with 45 mg/kg sodium pentobarbital and sacrificed by decapitation.

2.2. Histopathological examination

The animals were euthanized; their kidneys were collected and kept in 10% neutral buffered formalin for 48 h and then embedded within paraffin. Sections of 4 μm thickness were prepared using a rotary microtome. The sections were stained with hematoxylin and eosin (H and E). The morphological characteristics were assessed by light microscopy.

2.3. Apoptosis assay

Apoptosis in kidney cells was analyzed using a method that was described previously (14). The TUNEL assay was done by the in situ cell death detection kit (Beijing Zhongshan Biotechnology Co. Ltd, Beijing, China) according to the manufacturer's instruction. Paraffin wax-embedded tissue sections were treated with proteinase K (Sigma), and endogenous peroxidase activity was blocked by incubating with hydrogen peroxide. The sections were incubated at 37 °C for 60 min followed by rinsing the slides with phosphate-buffered saline (PBS). Nuclear labeling was developed with horseradish peroxidase and diaminobenzidine. Counterstaining was achieved by hematoxylin. When viewed under a light microscope, apoptotic nuclei were stained brown and nonapoptotic nuclei were blue. Apoptosis rate % = number of apoptotic cells/total cells × 100%.

2.4. *Bcl-2* and *Bax* assay

Immunohistochemical assay of *B*cl-2 and *B*ax assay were performed as described previously (15,16). Briefly, kidney tissues were fixed in 4% formaldehyde for 24 h followed by embedding in paraffin, and then cut into 4-μm slices using a microtome. The streptavidin peroxidase (SP) method was employed. The *B*cl-2 monoclonal antibody (Beijing, China) (1:50) or *B*ax polyclonal antibody (Beijing, China) (1:50) was incubated with the slices overnight at 4 °C or for 60 min at room temperature. The slices were observed and photographed under a light microscope and positive cytoplasm cells were stained yellow-brown.

2.5. Statistical analysis

Apoptosis was expressed as mean ± standard deviations (n = 10). The results were analyzed by Student's t-test and one-way analysis of variance (ANOVA). The effect was considered significant when the P value was < 0.05. All statistical analyses were performed using SPSS 17.0 (Released Aug. 23, 2008), Chicago, IL, USA.

3. Results

3.1. Histological results

The kidneys of the control group rats showed normal histological structure of glomerular and tubular structures (Figure 1A). Kidney tissues in the CsA treatment model group had significant morphological alterations compared to those of the control group, including tubular swelling and protein casts (Figure 1B). The kidneys of the RG-treated (0.2 mg/kg) rats showed focal tubular swelling and protein casts (Figure 1C), while the kidneys of those medicated with RG (0.4 mg/kg) showed no obvious histopathological change, and the harm to the kidneys was reversed in the RG-treated (0.4 mg/kg) groups (Figure 1D).

3.2. Immunohistochemical analysis

Immunohistochemical analysis demonstrated that the control rats had little kidney tubular cell apoptosis (Table; Figure 2A). In contrast, significant apoptosis was observed in the group treated with CsA (Table; Figure 2B). RG (0.2 mg/kg) decreased kidney tubular cell apoptosis in the CsA-treated rats ($P < 0.05$) (Table; Figure 2C). When the dose of RG was increased to 0.4 mg/kg, its inhibitory effect on CsA-induced apoptosis was more pronounced ($P < 0.01$) (Table; Figure 2D).

The expression of *Bax* protein in renal tubular cells in the CsA group was higher compared with that in the control group (Figure 3B), while *Bcl*-2 protein was obviously lower than in the CsA group (Figure 4B). The RG-treated (0.2 mg/kg) group had slight effect on the changes in the protein expression of *Bax* and *Bcl-2* induced by CsA (Figures 3C and 4C) while that of RG at a higher dose (0.4 mg/kg) had a more dramatic effect (Figures 3D and 4C).

4. Discussion

Apoptosis, or programmed cell death, has an essential role in development but also is the basis of many tissue lesions. It is regulated by a wide variety of factors, especially those belonging to the *Bcl-2* family: inhibitors (i.e. *Bcl-2* protein) and promoters (i.e. *Bax* protein) (17). Apoptosis regulator *Bax*, also known as *Bcl-2*-like protein 4, is a protein that is encoded by the *Bax* gene in humans. *Bax* is a member of the *Bcl-2* gene family. *Bcl-2* family members form hetero- or homodimers and act as anti- or proapoptotic regulators that are involved in a wide variety of cellular activities. This protein forms a heterodimer with *Bcl-2*

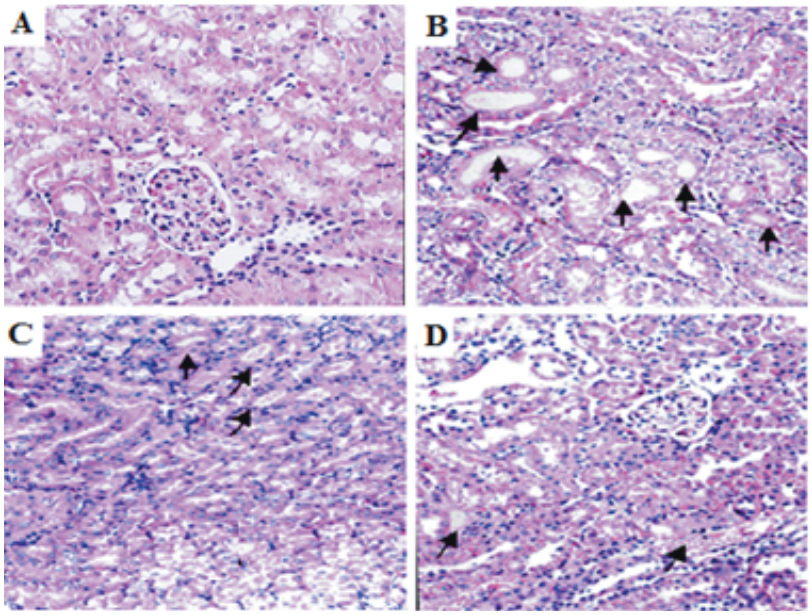

Figure 1. Light microscopy of kidney tissues from rats (HE stained kidney sections, 400×) (A) The control group: normal renal tubules. (B) The CsA group: tubules show extensive and marked swelling and protein casts (arrows). (C) The RG (0.2 mg/kg) + CsA group: tubules show swelling and obvious decrease in protein casts (arrows). (D) The RG (0.4 mg/kg) + CsA group: tubules show only a few and slight protein casts (arrows).

Table. Effect of repaglinide on cyclosporine A-induced renal tubule apoptosis. Data are expressed as mean ± SEM.

Group	Apoptosis rate/%
Control	0.32 ± 0.14
CsA (30 mg/kg)	7.62 ± 4.20**
RG (0.2 mg/kg) + CsA	5.24 ± 0.87#
RG (0.4 mg/kg) + CsA	0.68 ± 0.26##

Data given are the mean ± standard deviations (n = 10).
**Significantly different from the control group ($P < 0.01$).
#Significantly different from cyclosporine A group ($P < 0.05$).
##Significantly different from cyclosporine A group ($P < 0.01$).

and functions as an apoptotic activator. This protein is reported to interact with, and increase the opening of the mitochondrial voltage-dependent anion channel (VDAC), which leads to loss in membrane potential and release of cytochrome c (18). Previous studies showed that increased *Bcl-2* expression overall correlated with better survival rates, while overexpression of *Bax* played a role in the assessment of sensitivity for therapy (19,20).

Apoptosis plays an important role in the normal function and development of the kidneys. The apoptotic process within the kidney during organ development and during regeneration of tubules following renal damage helps appropriate nephron development and tubular repair after damage (21). Previous reports showed that renal diseases that have a dysregulated apoptotic process often cause loss of kidney function, abnormal development, or accelerated growth. The mitochondria pathway combines stress and developmental apoptotic factors, and this process is triggered by translocation of a proapoptotic *B*cl-2 family member such as *B*ax into mitochondria (21,22).

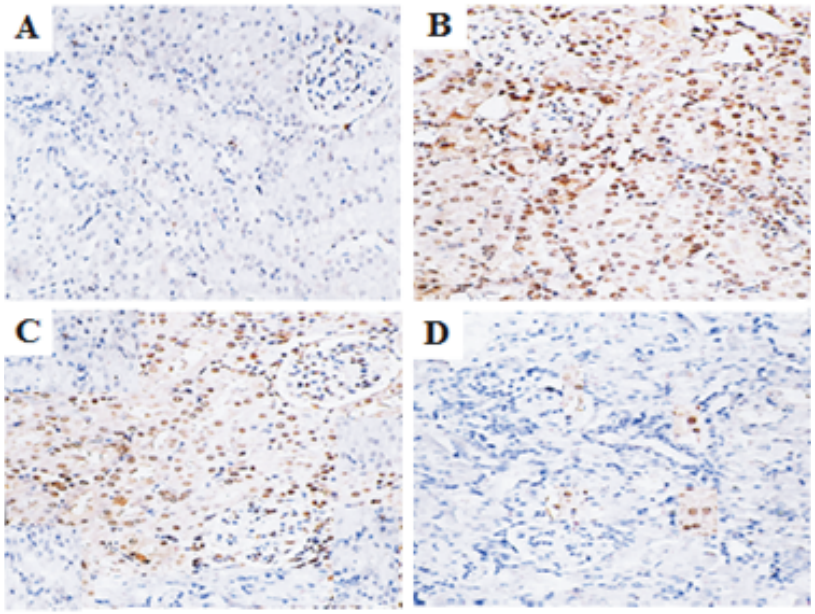

Figure 2. Effect of repaglinide on CsA-induced kidney tubular cell apoptosis (TUNEL, 400×). Apoptotic nuclei stained brown, nonapoptotic nuclei stained blue. (A) The control group: no kidney tubular cell apoptosis. (B) The CsA group: obvious kidney tubular cell apoptosis. (C) The RG (0.2 mg/kg) + CsA group: less kidney tubular cell apoptosis. (D) The RG (0.4 mg/kg) + CsA group: little kidney tubular cell apoptosis.

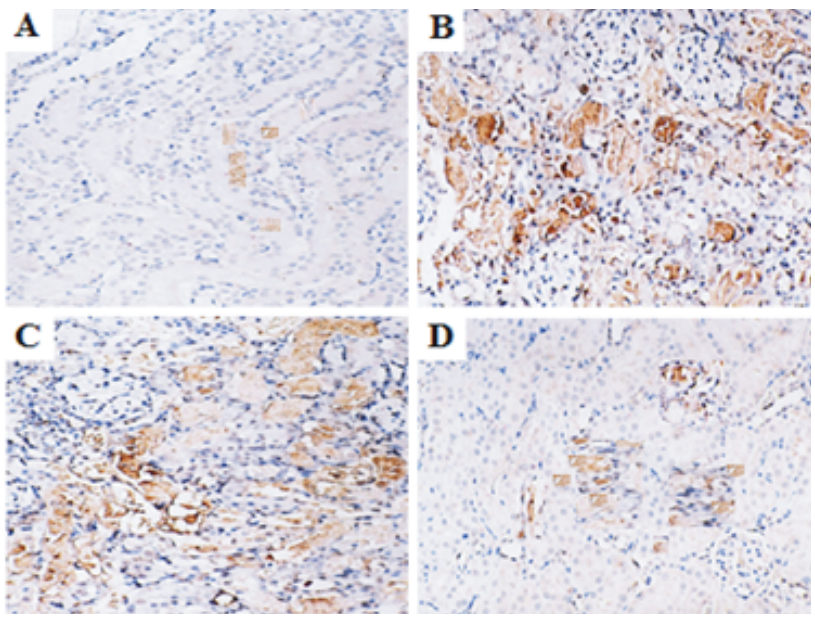

Figure 3. Effect of repaglinide on CsA-induced changes in the expression of kidney tubular cell *Bax* protein (SP, 400×). Cytoplasm was stained brown-yellow in *Bax* protein expression. (A) The control group: *Bax* was minimally expressed. (B) The CsA group: *Bax* expression was abundant. (C) The RG (0.2 mg/kg) + CsA group: *Bax* expression was weak. (D) The RG (0.4 mg/kg) + CsA group: *Bax* expression was very weak.

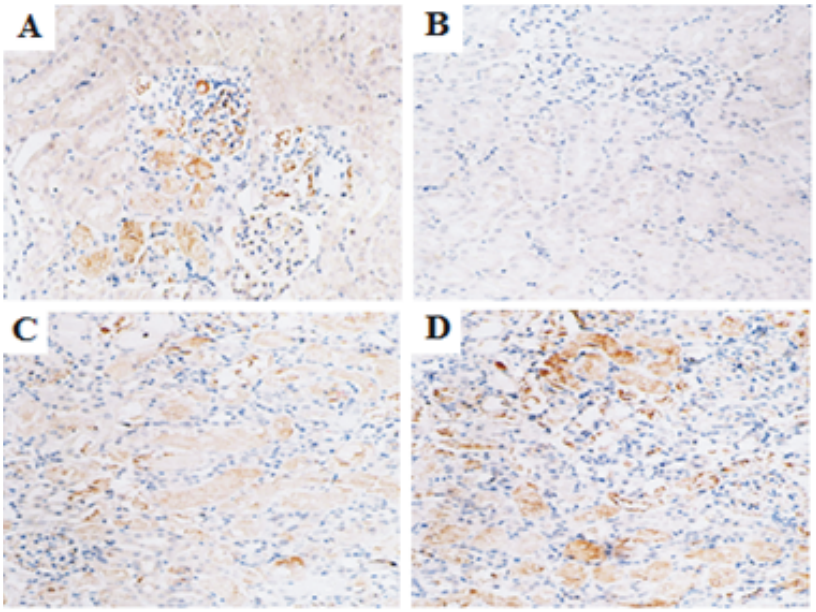

Figure 4. Effect of repaglinide on CsA-induced changes in the expression of kidney tubular cell *Bcl-2* protein (SP, 400×). Cytoplasm was stained brown-yellow in *Bcl-2* protein expression. (A) The control group: *bcl-2* expression was abundant. (B) The CsA group: little *bcl-2* expression. (C) The RG (0.2 mg/kg) + CsA group: *bcl-2* expression was weak. (D) The RG (0.4 mg/kg) + CsA group: expression of *bcl-2* was very abundant.

For example, CsA-induced cytotoxicity was related to apoptosis in tubular or endothelial cells (12,13). However, the apoptotic pathway can potentially be modulated to maintain cell viability. Moreover, the components of the apoptotic pathway are amenable to therapeutic modulation (23).

In the current study, we present results of an immunohistochemical analysis of renal tubular cell apoptosis and apoptosis-associated protein expression in CsA-induced renal tubular injury. Our study showed that CsA induced significant apoptosis in renal tubular cells. Moreover, CsA significantly increased the expression of *Bax* protein and decreased the expression of *Bcl-2* protein. Our findings are in accordance with previous reports where CsA induces kidney tubular cell apoptosis (12,13). RG significantly inhibited renal tubular cell apoptosis and increased the expression of *Bcl-2* protein and reduced the expression of *Bax* protein in CsA-induced renal tubular toxicity. Therefore, RG effectively reverses CsA-induced renal tubular toxicity and this may be related to the inhibition of renal tubular cell apoptosis. The present study provides evidence that RG may have certain degrees of renoprotection by affecting apoptosis and expression of apoptosis-associated proteins. The mechanisms by which RG affects apoptosis need further study.

In conclusion, the results of our study indicate that CsA causes renal tubular toxicity by inducing apoptosis. RG may play a renoprotective role in CsA-induced renal tubular toxicity by suppressing apoptosis.

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